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# Effects of a novel recombinant somatostatin DNA vaccination on rat fertility and offspring growth

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**In this study, we investigated the immune effects of a novel somatostatin (SS) DNA vaccine—pVAX-asd-GS/2SS (pGS/2SS), administrated with intramuscular (IM) and subcutaneous (SC) two delivery routes on female rat fertility and offspring growth. Results show that this pGS/2SS DNA vaccine could induce effective anti-SS immune response in rats (IM group and SC group). The antibody peak of female rats in IM group occurred later than that in SC group (12th week vs. 10th week), but higher than that in SC group (OD=1.122±0.273 vs. OD=0.614±0.183). Immunized groups had higher pregnancy rate, litter size, birth weight of pup and weight gain of pup than the control group (P<0.05). Compared to SC immunization, IM immunization had better improvement in the pregnancy rate of dam and the weight gain of pup (P<0.05). However, in litter sizes and birth weight of pups, SC immunization was better than IM immunization. In conclusion, pGS/2SS as a powerful DNA vaccine improves the fertility of female rats and the growth of pups.**

**Key words:** Somatostatin, DNA vaccine, rat, fertility, pup growth.

## INTRODUCTION

Somatostatin (SS) is a tetradecapeptide, which is produced by neuroendocrine neurons of the periventricular nucleus of the hypothalamus and mammary gland (Reisine 1995; Lin et al., 1998). SS inhibits the secretion of most hormones (example, growth hormone, insulin, glucagon, thyrotropin, luteinizing hormone) closely related to the reproduction or production performance of animals (Julien et al., 2004; Van Vugt et al., 2004; Engin et al., 2008; Liang et al., 2008). Previous studies revealed that SS inhibited the secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), and there was a negative feedback regulation between SS and estradiol (Holloway et al., 1997; Cardenas et al., 2003; Pillon et al., 2004; Van Vugt et al., 2004). SS also

inhibited prolactin secretion in the existence of estradiol (Horváth and Schally, 1994; Lee and Shin, 1996). Chen et al. (1999) indicated that SS was the only neuropeptide that was found to be produced by the mammary gland of rat, and involved in the biology of the suckling neonate and probably also in the development and function of the breast. Growth hormone (GH) administration has been used to increase milk yield in ruminants for a long time (Flint et al., 1992), but GH secretion was inhibited by SS. These studies showed that SS could down-regulate the reproduction and milk secretion (closely related to the pup weight gain) of animals by inhibiting the secretion of different hormones. One hypothesis stated that the decreased SS level could improve the reproduction performance of animals and increased the weight gain of pups. Presently, immuno-neutralization of SS has been used in various animals (Kirkwood et al., 1990; Kim et al., 2001, 2002; Ren et al., 2003). However, purification process of SS antigen is very complicated and tedious, as well as costly and arduous. Additionally, SS antigen has low immunogenicity and cannot easily evoke an

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**Abbreviations:** SS, Somatostatin; SC, subcutaneous; IM, intramuscular

effective immune response. Recently, DNA vaccine has become a reliable and major mean to induce immune response in the past decade (Apostolopoulos and Plebanski, 2000; Leitner et al., 2000; Ada and Ramshaw, 2003; Donnelly et al., 2005). A DNA vaccine offers the advantages of easy preparation, high immunity, and low cost. A few kinds of SS DNA vaccines have been developed (Xue et al., 2007; Han et al., 2008; Liang et al., 2008, 2009; Bai et al., 2011). A novel recombinant SS DNA vaccine- pVAX-asd-GS/2SS (pGS/2SS) attracted more attention (Liang et al., 2009), as pGS/2SS DNA vaccine could immunoneutralize endogenous SS content and release some hormones inhibited. The GS/2SS fusion gene from the pGS/2SS DNA vaccine encoded two copies of somatostatin gene presented by the hepatitis B surface antigen (HBsAg) particle and granulocyte/macrophage colony-stimulating factor gene (GM-CSF). This GS/2SS fusion gene was expected to bring high immunogenicity. Most commonly used routes of administration in laboratory animals are intramuscular (IM), subcutaneous (SC), Intravenous (IV), Intraperitoneal (IP), orally and electroporation, etc. Different delivery routes could bring different immune effects. The aims of the current study were to verify whether the pGS/2SS DNA vaccine can induce an effective immune response and to improve the fertility of female rats and the growth of offspring, and two delivery routes (IM and SC) were compared.

## MATERIALS AND METHODS

### Preparation of pGS/2SS DNA vaccine

The construction of pGS/2SS DNA vaccine has been reported previously (Liang et al., 2009). The pGS/2SS plasmid included two copies of somatostatin genes and important enhanced immune factors: hepatitis B surface antigen (HBsAg) particle and granulocyte/macrophage colony-stimulating factor gene (GMCSF). These genes were fused and cloned into a pVAX-asd vector (Figure 1). The pGS/2SS-asd plasmid was transformed into *S. enterica* sv. *Choleraesuis* C500 strain and then dissolved in physiological saline as DNA vaccines for immunization.

### Animals and immunization

A total of 72 female Sprague-Dawley rats (five weeks of age, purchased from the Faculty of Laboratory Animal, Tongji Medical College of Huazhong University of Science and Technology, Hubei Province) were maintained in the Laboratory Animal Center, Huazhong Agricultural University. The animals were housed at 22±2°C at a relative humidity of 50±5%. The lighting regime was 14 h of light (from 0600 to 2000) and 10 h of dark. Food and water were supplied *ad libitum*. All experimental animals and procedures used in this study were in agreement with the rules of the Laboratory Animal Research Centre of Huazhong Agricultural University.

All rats were divided into three groups (24 rats per group) randomly. Two test groups were immunized with pGS/2SS: in intramuscular (IM) group, each rat was injected with 200 µL (10<sup>10</sup>CFU/mL); in subcutaneous (SC) group, each rat was also injected with 200 µL (10<sup>10</sup>CFU/mL). The third group was injected

with physiological saline (200 µl) as a control. The first week of the test (primary immunization) was considered as week 0. Booster immunizations twice with the same dosage were performed at four-week intervals (week four and eight).

### Collection of blood samples

Blood samples from female rats were collected from the tail vein with heparinized tubes at week 0, 2, 4, 6, 8, 10, 12, 13, 14, 15 and 16. Blood samples from rat pups at the time of weaning were also collected by tail vein. Following centrifugation at 4000×g for 5 min, the plasma was separated from the whole blood and stored at -20°C.

### Detection of antibody

An indirect enzyme linked immunosorbent assay (ELISA) method was carried out to measure serum specific SS antibodies in all groups. Somatostatin-14 (S9129, Sigma–Aldrich) was used as the standard antigen. Plasma samples were diluted 1:50. HRP-labeled goat anti-rat IgG antibodies (Boster, Wuhan, China) were used as a secondary antibody and 3, 3', 5, 5'-tetramethylbenzidine (TMB) was served as a substrate. Optical density was measured at 450 nm after addition of 0.5 M H<sub>2</sub>SO<sub>4</sub> to stop the reaction. In this study, the OD value was used to evaluate the antibody level. Any rat from all groups was considered as rat with positive immunization response if the OD value was as ≥3 times as those in the control group. On the contrary, the rats with the OD values <3 times were considered as rat with negative immunization response and the specific SS antibodies were under detectable.

### Measurement of rat fertility and offspring growth

Pregnancy rate, litter sizes of dams and birth weights of pups were used to measure the fertility of dams. Natural mating was used (male:female=1:3). Mating date was arranged at the second week after the last booster (week 10). A vaginal plug appeared was designated as pregnancy. Pregnancy rates of dams during an estrous cycle were recorded. Data of litter sizes and birth weights of pups after parturition were collected.

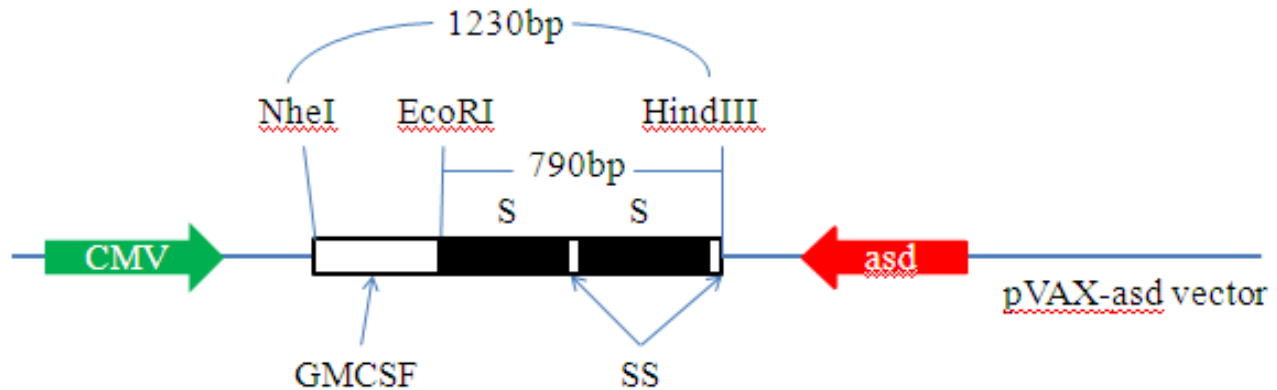
To assess the possible effect of vaccination on the growth of pup during lactation, weight gains of pups from birth to weaning were considered. After parturition, the litters were culled to eight pups per dam and gender was balanced if possible within 24 h. There were no statistical differences for birth weights of the pups among 3 groups (P>0.05). All pups were allowed to suckle during the first 3 weeks after parturition.

### Analysis of hormone

The levels of growth hormone (GH) in offspring were detected by radio-immunoassay (RIA). Serum concentrations of GH in offspring at the time of weaning were measured by RIA kit (Beijing Chemclin Biotech Co. Ltd, Beijing, China). The intra- and inter- assay coefficients of variation for GH were <15 and <10%, respectively. The sensitivities for the GH assays were ≤0.5 ng/mL.

### Statistics

The pregnancy rate of dams in different immunized groups and the difference between animals with or without antibodies were analyzed by Chi-square test. One-way ANOVA was used to analyze other data including litter sizes of dams, birth weights of pups, GH concentrations of pups, weights at birth, weaning, and weight gains



**Figure 1.** Structure of the plasmid pGS/2SS. The CMV enhancer promoter regulatory region drove the expression of the pGS/2SS. The fused gene consisted of GMCSF gene and two somatostatin fragments (SS) linked to HBsAg(S), was subcloned into a pVAX-asd vector.

of the pups (8 pups per dam) during lactation period. All data were reported as the means $\pm$ S.D. A difference was considered significant at the  $p < 0.05$  level.

## RESULTS

### Identification of pGS/2SS plasmid

The pGS/2SS plasmid was doubly digested. There were a GS/2SS segment (1230 bp) with NheI and HindIII digestion (Lane 2, Figure 2) and an S/2SS segment (790 bp) with EcoRI and HindIII digestion (Lane 3, Figure 2) in the electrophorogram. The pVAX-asd vector (control) was also digested with NheI and HindIII enzyme (Lane 1, Figure 2).

### SS antibody levels and positive detection rate of dams and pups

The SS-specific antibodies were detectable in IM group and SC group, while no antibodies were detectable in the control group. During the first four weeks after primary immunization, antibody levels were very low in all immunized dams. However, the specific antibody levels increased in a time-dependent manner in IM group and SC groups after the first booster (after week 4), and reached the peak level at week 10 in SC group (OD=0.614 $\pm$ 0.183) or week 12 in IM group (OD=1.122 $\pm$ 0.273), respectively and then decreased until 16 weeks (Figure 3). Antibody levels of dams were analyzed at three periods (pre-mating, post-parturition, and post-weaning). At the periods of pre-mating and post-parturition, the positive signal of antibodies was always at 100% in IM group and SC group, but at the time of weaning, the positive rate of IM group was obviously higher than that of SC group ( $P < 0.01$ , Table 2).

The SS-specific antibodies of pups in IM group and SC group were also detectable after weaning, and evidently

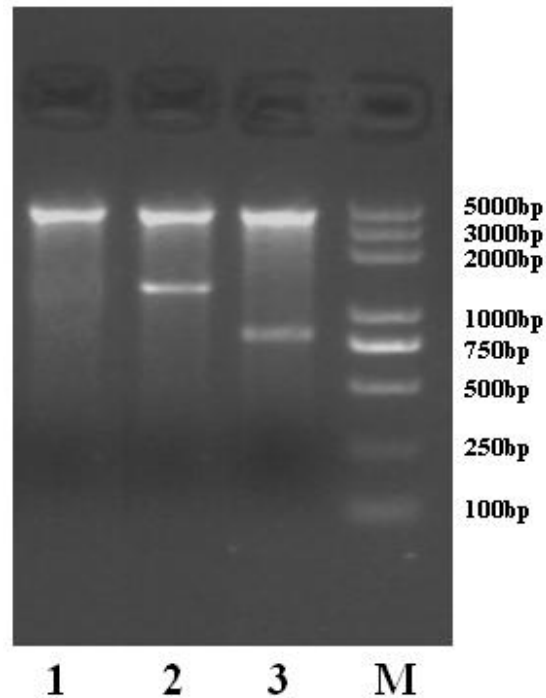
higher than those in the control group ( $P < 0.01$ , Figure 4). Antibody levels of pups in IM group were higher than those in SC group (OD=0.620 $\pm$ 0.349 vs. 0.362 $\pm$ 0.156,  $P < 0.01$ , Figure 4). The antibody positive rate of pups in IM group after weaning was significantly higher than that in SC group ( $P < 0.01$ , Table 2).

### Pregnancy rate and litter sizes of dams and birth weights of pups

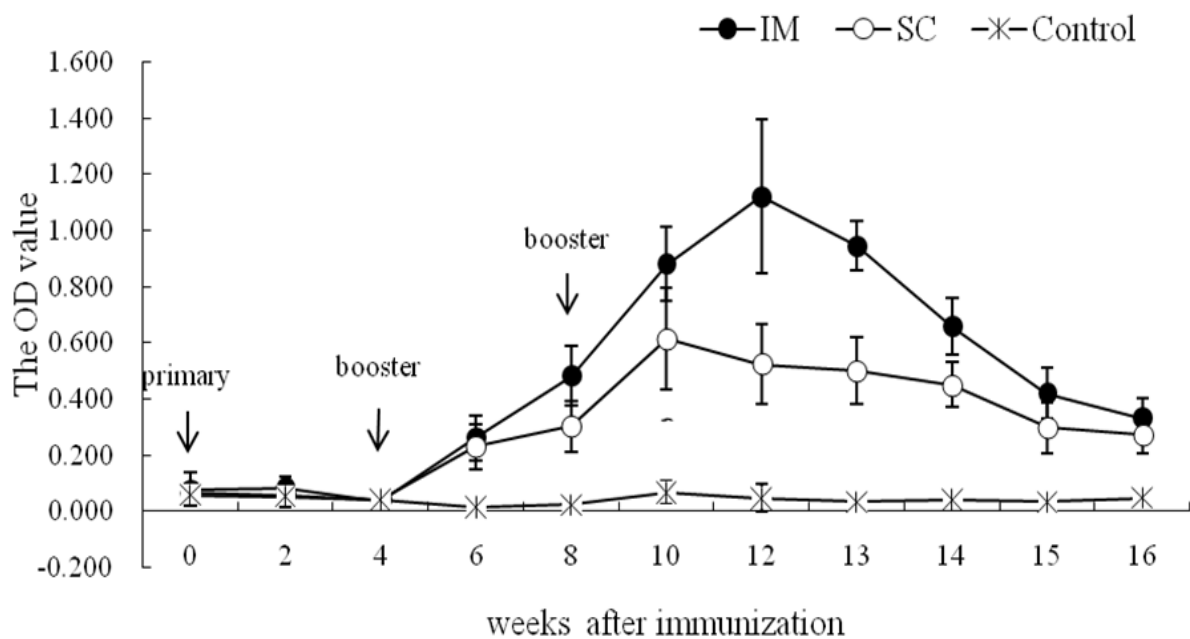
The pregnancy rates of dams in IM group and SC group were both higher than that in the control group ( $P < 0.05$ , Table 3). The pregnancy rate of dams in IM group was higher than that in SC group, but the difference is not significant (Table 3). Litter sizes of dams in IM group and SC group were both higher than those in the control group, and the difference was significant between SC and control group ( $P < 0.05$ , Table 3). Birth weights of pups in IM group and SC group were significantly greater than those in the control group ( $P < 0.05$ , Table 3). Though Birth weights of pups in IM group were higher than those in SC group, there was no significant difference between IM group and SC group.

### Weight gain and GH concentrations of pups after weaning

Weight gain of pups (8 pups per dam) from birth to weaning was used to measure the growth of pups during lactation. There were no significant differences in birth weights among all groups. Pups in IM group and SC group have the higher weight and weight gain than those in the control group at the time of weaning ( $P < 0.05$ ). Pups in IM group had higher weight and weight gain than those in SC group ( $P < 0.05$ , Table 4). There was no significant difference among all pups of three groups in GH concentrations (Table 4).



**Figure 2.** Identification of pGS/2SS. Lane1, pVAX-*asd* vector (3760bp, pVAX-*asd* vector was digested with *Nhe*I and *Hind*III enzyme); Lane 2, GS/2SS fusion gene (1230 bp, pGS/2SS plasmid was digested with *Nhe*I and *Hind*III enzyme); Lane 3, S/2SS fuse gene (790 bp, pGS/2SS plasmid was digested with *Eco*RI and *Hind*III enzyme); M, DL5000 DNA marker.



**Figure 3.** SS-specific antibody levels (OD value) administrated by paGS/2SS with two routes (IM and SC) in female rats. Rats were injected at five weeks of age. In the first 4 weeks after injection, the level of antibodies was very low, and then increased after the first booster immunizations, and reached the peak level at 10 weeks (SC, OD=0.614±0.183) or 12 weeks (IM, OD=1.122±0.273) respectively, and then declined until 16 weeks.

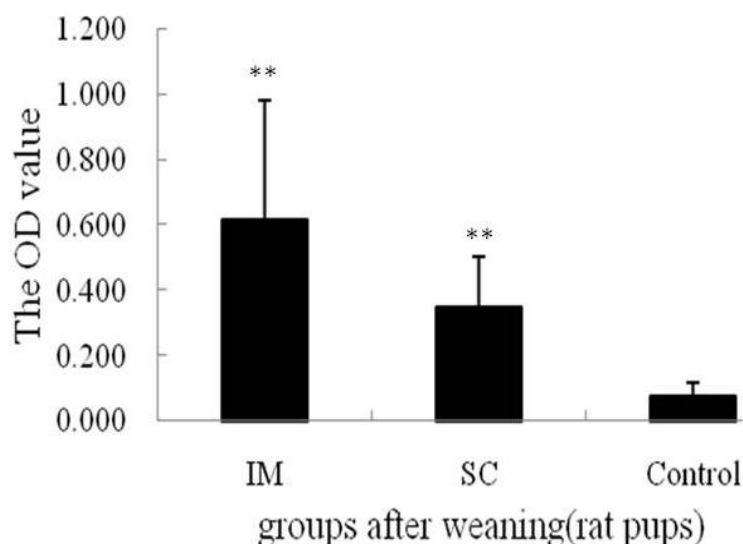
**Table 1.** The primer sets for PCR in this study.

Primer name	Sequences of primer	Anneal temperature (°C)	Fragment length (bp)
P <sub>β-actin</sub>	F:CGT TGA CAT CCG TAA AGA CC R:AAC AGT CCG CCT AGA AGC AC	55	270
P <sub>GS/2SS</sub>	F:GCT GGC TAG CTC AGA AGG AT R:AGC GGT ACC AAC GTA TAC CC	55	1230

**Table 2.** Antibody positive detection rate of dams and pups at pre-mating, post-parturition, and post-weaning periods.

Index	Dam			Pup
	Pre-mating	Post-parturition	Post-weaning	Post-weaning
IM	100.0%(24/24)	100.0%(23/23)	78.3%(18/23)**	75.0%(138/184)**
SC	100.0% (24/24)	100.0% (22/22)	31.8%(7/22)	58.3%(103/176)

\*\* P&lt;0.01.

**Figure 4.** SS-specific antibody levels of pups after weaning. Antibody levels of pups of two test groups after weaning were significantly higher than those of the control group (P <0.01), with the highest level of antibodies in IM group (P <0.01). \*\* means P<0.01.

## DISCUSSION

In present study, SS-specific antibodies of female rats immunized with two routes (IM and SC) were detectable by an indirect ELISA. This novel pGS/2SS vaccination could induce SS-specific antibody maintained for 10 weeks. This result was obviously different from a previous report (Liang et al., 2008). Liang et al. (2008) showed that the peak level of antibodies occurred at week 2 after primary injection with pcS/2SS plasmid. The vaccination in the present results could induce higher antibody level than that in Liang's study. However, the emergence of the

immune response was slow (about 6 weeks after immunization). The possible consideration was that the new presentation system (pVAX-*asd* vector+GS/2SS fuse gene, Liang et al., 2009) delayed the immune system activation. The regulatory mechanism of the new presentation system *in vivo* is now still unclear. Further study is expected to provide clarifications or solutions to these concerns.

In this study, two delivery routes were used. The antibody peak of dams in IM group occurred later, but higher than that in SC group. At the period of pre-mating and post-parturition, positive signals of antibody were

**Table 3.** Pregnancy rate, litter sizes of dams and birth weights of pups in all groups.

Group	Pregnancy rate (%)	Litter size (n)	Birth weights of pup (g)
IM	95.8 (23/24) <sup>a</sup>	12.7±2.3	79.5±10.5 <sup>a</sup>
SC	91.7 (22/24) <sup>a</sup>	13.5±2.2 <sup>a</sup>	80.2±10.3 <sup>a</sup>
Control	83.3 (20/24) <sup>b</sup>	11.8±2.3 <sup>b</sup>	73.7±9.1 <sup>b</sup>

Different letters in the same column mean significantly different at 0.05 levels ( $P < 0.05$ ).

**Table 4.** Birth weights, weaning weights, weaning weight gains and GH concentrations of pups in three groups (8 pups per dam).

Groups	Birth weight (g)	Weaning weight (g)	Weaning weight gains (g)	GH (ng/mL)
IM	49.7±0.6	352.6±14.3 <sup>a</sup>	302.9±14.1 <sup>a</sup>	1.1 ±0.2
SC	49.5±0.3	343.9±13.9 <sup>b</sup>	294.4±13.9 <sup>b</sup>	1.0 ±0.3
Control	49.7±0.3	335.5±11.7 <sup>c</sup>	285.9±11.8 <sup>c</sup>	1.1 ±0.4

Different letters in the same column mean significantly different at 0.05 levels ( $P < 0.05$ ).

detectable in both IM group and SC group, but the difference was not significant between two groups. At the period of post-weaning, the positive rate of antibodies in IM group was significantly higher than that in SC group. These results suggested that IM and SC vaccine deliveries could induce antibody responses and IM route had a good advantage over SC route.

SS-specific antibodies of pups after weaning were also detectable in both IM group and SC group. It seemed that there is a possible correlation in the antibody levels between dams and their pups. It was predicted that the high SS-specific antibody level of dams evoked the existence of pup SS-specific antibody. The exact mechanism of pup antibody is still unknown. In addition, we examined the GH secretion of the weaned offspring, but the differences of offspring in all groups are not significant in GH levels. It indicated that antibodies induced *in vivo* did not affect GH levels. GH pulse in the body is released and SS-specific antibody is induced by the immune system *in vivo*. The possible explanation is that in order to maintain the normal state of animal body, the balance between GH and SS antibody is adjusted. Further targeted experiments can explain these results.

A close correlation exists between the somatotrophic and gonadotropic axes, where either underproduction or overproduction of GH can affect reproduction in animals. The GH secretion was inhibited by SS. Previous reports confirmed that SS could inhibit the secretion of LH and FSH, and prevent folliculogenesis and enhance atretic processes in rat (Nestorović et al., 2001, 2004). Bai et al. (2011) showed that oral administration with somatostatin DNA vaccine (pGM-CSF/SS) had a significantly shorter estrous cycle than controls, which was in agreement with the significantly higher level of  $E_2$  on the day of parturition. These studies imply that if the SS level of rat was decreased, follicular development and ovulation could be improved. Therefore, it is believed that SS is

involved in the regulation of reproductive function in animals. In a previous study (Kirkwood et al., 1990); ewes and pigs were actively immunized against a SS-ovalbumin conjugate. Although no difference was seen in the pregnancy rate between immunized and control pigs, pregnancy rate was higher for immunized than control ewes. In the current study, pregnancy rates of IM and SC groups were significantly higher than that in the control group; the pregnancy rate of IM group was the highest among these three groups. This result shows that SS immunization did improve pregnancy rate of rats. Litter sizes of dams in IM and SC groups were more than those in the control group, which was similar to the above results in which the first litter size tended to be larger in immunized pigs with SS-ovalbumin conjugate than that in the control pigs (Kirkwood et al., 1990). The roles of SS vaccination for birth weights of pups were not similar in different animals (Sun et al., 1996; Van Kessel et al., 1990). The current results reveal that birth weights of pups in IM group and SC group were significantly higher than those in the control group. Furthermore, at the period of pre-mating to post-parturition, positive signals of antibody in IM group and SC group were always detectable. These results confirm that the development of pups during pregnancy could be improved by SS vaccination. Regarding the results of litter sizes of dams and birth weights of pups, SC delivery was better than IM delivery.

Some studies showed that active or passive immunization against SS could not increase the milk production of rat (Sun et al., 1996; Yi et al., 1999). However, SS was considered the only neuropeptide that was produced by the mammary gland (Ren et al., 2003). It was shown that SS inhibited prolactin (PRL) secretion in the participation of estradiol (Kim et al., 2001, 2002). Growth hormone was also involved in milk secretion (Leitner et al., 2000). Sun et al. (1997) considered that

passive immunization of SS improved growth rate of litters and milk yield in rats. So the prediction was considered to be that the decreased SS level would lead to the release of GH and PRL, and improve milk yield in animals. Due to the close relationship between milk yield and weight gain of pup during lactation, it is possible to improve puppy growth during lactation by genetic immunization. Though the GH levels of the weaned offspring are not different, Pup weight gains in both IM group and SC group were significantly higher than those in the control group. Furthermore, SS-specific antibodies of pups in these two groups after weaning were also detectable. It suggested that the immuno-neutralization by the pGS/2SS vaccine improved puppy growth during lactation. IM delivery has an advantage over SC delivery at this point.

In conclusion, we demonstrated that this novel DNA vaccine had a good immunogenicity and could induce high immune response in female rats. Immunization against pGS/2SS not only improved the pregnancy rate, litter size and the birth weight of pup but also increased the growth of pup during lactation. Compared to SC immunization, IM immunization produced a higher antibody level and had a better improvement in the pregnancy rate of dam and the weight gain of pup. However, in litter sizes and birth weight of pups, SC immunization was better than IM immunization. Further studies were put on to explore the mechanism of this vaccine *in vivo*.

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